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The role of estradiol (E2) metabolism in mammary carcinogenesis is being studied in a rat model. Two strains are employed: the ACI is extremely sensitive to E2 carcinogenicity, whereas the Sprague-Dawley is resistant. Characterization of E2 metabolism in liver microsomal preparations showed robust metabolism from 60 μM down to 3 nM. The latter value approximates the physiological concentration of E2. In the micromolar range both strains produce estrone and 2-hydroxy-E2. In the nanomolar range, however, estrone is the dominant product from the Sprague-Dawley whereas the ACI makes almost entirely 4-hydroxy-E2. Given that 4-hydroxy-E2 may be the key carcinogenic metabolite of E2, this strain-specific metabolism may explain the noted difference in E2 carcinogenicity between the two strains. Although liver determines the systemic fate of E2, metabolism and effects of E2 in target tissues are of even more concern. Therefore, these studies will be extended to the rat mammary epithelial cell (RMEC) system. We have isolated and cultured RMEC from both rat strains. The resulting cultures are, a mixed population, containing about 65% myoepithelial cells surrounding colonies of lumenal epithelial cells. Although metabolism will provide a summation of contributions from all cell types, the studies of microtubule effects and aneuploidy will be cell-type specific.

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Johnny A Reed 7/10/98

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Introduction

The identification of carcinogenic risks may be derived from two complementary approaches: the first is epidemiological analysis of human populations, while the second is by extrapolation from controlled exposure studies with experimental animals. The association between estrogens and breast cancer has been supported by ample data from both approaches. In humans, clinical and epidemiologic data provide provocative links between the length and level of estrogen exposure and the incidence of breast cancer. Prenatal exposure to the synthetic stilbene estrogen diethylstilbestrol (DES) is associated with an increased incidence of malignancy at several sites, including the breast (1,2). Exposure to the estrogens estradiol (E2) or estrone (E1) also is associated with an increased incidence of breast cancer (3). Early menarche and late menopause each will lengthen the exposure period to endogenous estrogens, and each is considered a risk factor for breast cancer in humans (4). The use of high-dose estrogens as "morning-after" contraceptives further enhances estrogen exposure, as does the common post-menopausal hormone replacement therapy. Although links between estrogens and human breast cancer are apparent, determination of the mechanism of action is required before the magnitude of these risk factors can be determined.

Animal studies of estrogens and carcinogenesis provide more direct and quantifiable evidence for a causal role in tumorigenesis (Reviewed in 2). E1, E2 , and DES induce mammary gland tumors in both rats and mice, and estriol is also a mammary carcinogen in mice. The synthetic steroids norethynodrel and mestranol are mammary carcinogens in rats and dogs, respectively. In addition to these effects in mammary gland, estrogens also are carcinogenic in the liver, kidney, pituitary, and in various organs of the genitourinary tract of several species. Induction of tumors in multiple tissues and species strongly supports the IARC classification of estrogens as human carcinogens (1).

Despite the clear association between estrogens and breast cancer, the mechanisms involved in this effect are not clear. Conceptualization of estrogen carcinogenicity has taken several forms. The first is that estrogens, as one of their normal activities, promote proliferation of mammary epithelial cells (2,3,5). Since the process of DNA replication is not accomplished with absolute fidelity, each replication cycle carries an infinitesimal but finite risk of errors, resulting in point mutations. Increased rate and extent of mammary epithelial cell proliferation, induced by estrogens, is proposed to increase not only the general mutation frequency but also the frequency of specific mutations in proto-oncogenes or tumor suppressor genes, resulting in malignant transformation. Although this seems a straightforward mechanism based on the well-known actions of the hormone, it would seem that the probability of such specific mutations occurring would be extremely small. A more compelling mechanism would include both the proliferative effects of estrogens and a genotoxic insult.

Such a mechanism has been proposed, in which estrogens also function as classical genotoxic carcinogens (6). Either the estrogen itself or an activated metabolite is able to react with DNA, resulting in the formation of DNA adducts, which then lead to point mutations or other structural and functional alterations in DNA. It is the accumulation of specific changes in specific genes which leads to the malignant phenotype. Although a great deal of evidence has been presented regarding the formation of estrogen-DNA adducts both *in vitro* and *in vivo*, these data are not compelling. Nucleoside and nucleotide adducts generated from DES and other estrogens *in vitro* have been isolated and characterized. However, these adducts are formed at levels well below those achieved with typical genotoxic carcinogens, and the formation of such adducts *in vivo* required a dose of DES orders of magnitude above a tumorigenic dose (7).

A variation on this proposed genotoxic mechanism is that estrogens and their metabolites are indirectly genotoxic. That is, estrogen metabolites such as catechols and hydroquinones are able to redox cycle, and thus produce large amounts of reactive oxygen species. It then is suggested that these ROS, via direct oxidation or hydroxylation of DNA, produce promutagenic lesions, leading to point mutations and contributing to a malignant phenotype. Again, the ability to cause such DNA damage *in vitro* has been well documented (8,9), however the presence of oxidative DNA damage resulting from *in vivo* exposure has not been reported.

An intriguing proposal for the mechanism of estrogen carcinogenesis involves the generation of reactive

metabolites from the estrogen, but then invokes the covalent modification of proteins, rather than DNA. Many estrogens or their hydroxylated metabolites are hydroquinones or catechols. Such compounds readily autoxidize or may be enzymatically oxidized to their corresponding semiquinone radicals or quinones (10-12). Tautomerization to quinone methides also is documented (13,14). Quinones and quinone methides are particularly susceptible to attack by sulfur and other heteroatoms, and the heteroatom-containing amino acid side chains of proteins are predominant sites of covalent binding (15-17). The relevance of this protein binding to carcinogenesis has been discounted as it is difficult to link modification of proteins with the induction of heritable changes in cellular control mechanisms. This link, however, has been made based on the ability of DES and other estrogens to bind to tubulin (18,19). Peroxidative oxidation of DES and of E2 catechols, which generates quinones and semiquinones, resulted in extensive covalent modification of protein in the presence of tubulin. Parallel studies of estrogen oxidation in the presence of bovine serum albumin yielded only a fraction of the protein binding seen with tubulin, pointing to the specificity of the interaction. This covalent modification blocks and reverses tubulin polymerization, and thus corresponds to the known ability of DES and other estrogens to disrupt microtubules, both in isolated form (18-20) and in cultured cells (21-24).

The consequences of the disruption of microtubules during mitosis are well appreciated. Complete loss of mitotic microtubules results in metaphase arrest, as no mechanism remains for the segregation of sister chromatids into progeny cells. Lesser disruption of mitosis, however, can be even more damaging to the organism. Partial disruption of chromosomal segregation leads to aneuploidy, as the distribution of chromosomes between daughter cells is not equal. Unlike metaphase arrest, such chromosomal aberrations may result in cells with altered genotypes that nonetheless are proliferation competent, thus amplifying the altered genotype. The induction of aneuploidy by DES (22,23,25-27) and other estrogens (24,28) has been observed in several mammalian cell types *in vitro*. The importance of the induction of aneuploidy in mammalian cells is supported by the concurrent emergence of cell transformation (25,27,28). Further study of DES-induced aneuploidy and transformation in Syrian hamster cells demonstrated that the immortalization and tumorigenicity of the resulting cells correlated with non-random karyotypic changes (29). The analysis of these non-random chromosomal changes, and particularly of the specific genes either lost or duplicated, will provide powerful insights into the changes in growth and differentiation comprising the malignant phenotype.

In order to advance the understanding of microtubule disruption and aneuploidy in estrogen-induced mammary carcinogenesis, two approaches are required. One is to determine if the effects on microtubule integrity *in vitro*, demonstrated convincingly in various cell lines, will also occur in mammary epithelial cells, the target cell population for tumorigenicity. Such an investigation also should include the careful examination of the mechanism of the effects, and should look for specific chromosomal aberrations which might serve as heritable biomarkers of this effect. The second approach will be to look for microtubule disruption and chromosomal aberrations in mammary epithelial cells resulting from treatment with tumorigenic doses of estrogens *in vivo*. Although the latter approach is required for the most definitive answer to the question of mechanism of estrogen tumorigenicity in the mammary gland, the *in vitro* approach provides a much more efficient system for the initial study of this mechanism in the relevant cell population. The *in vitro* studies also may elucidate the key biomarkers to then apply in the *in vivo* experiments. For these reasons, we propose to carry out a systematic, stepwise examination of the fate of estrogens in mammary epithelial cells, and the resultant ability of those compounds to affect microtubule integrity and to ultimately induce chromosomal aberrations. This will provide the first examination of this intriguing mechanism for malignant transformation by epigenetic means in mammary epithelial cells.

This proposed mechanism differs markedly from that of another mammary carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA). Most evidence supports the metabolism of DMBA to diol epoxide or other reactive intermediates which then covalently modify DNA by forming adducts, leading to point mutations (30,31). This means that the production of specific stable metabolites of DMBA and the formation of specific DNA adducts should be hallmarks of sensitivity to DMBA carcinogenesis, whereas tubulin modification by DMBA or the disruption of microtubules should not be observed.

A pronounced strain difference in sensitivities to mammary carcinogenesis by these two classes of agents also will be exploited in these studies. The ACI rat is sensitive to estrogens as mammary carcinogens (32-35), but is quite resistant to the effects of DMBA and other classical genotoxic carcinogens (34,36). Sprague-Dawley rats, in contrast, exhibit the reverse pattern of sensitivity (33,37). By examining the metabolism and resultant effects of both classes of carcinogen in both strains it not only is possible to look for differences in metabolism and responses, but also to try and correlate these responses with susceptibility to carcinogenesis. Our expectation is that ACI rat mammary epithelial cells will effectively convert E2 to forms which disrupt microtubules and cause aneuploidy, but which do not form DNA adducts. We expect the ACI rat cells to exhibit little ability to activate DMBA and generate DNA adducts. In the case of the Sprague-Dawley, we expect little or no activation of E2 or induction of aneuploidy, but do expect that DMBA-derived DNA adducts will be readily apparent. Such a result would underscore the two different mechanisms of attack on genomic integrity of mammary epithelium, and would clearly link each mechanism to its respective chemical class of initiator and to the observed sensitivity or resistance to carcinogenesis by these two classes of agents.

Experimental Methods

Materials: [^{3}H]- and [^{14}C]-Estradiol were products of New England Nuclear. Estradiol (E2), estrone (E1), and their hydroxylated metabolites, DNase, clotrimazole, and all other substrates and cofactors were from Sigma Chemical Co. (St. Louis, MO). Balanced salt solutions and media were purchased from GIBCO/BRL. Percoll is a product of Pharmacia. Collagenase (Type I) was from Worthington Biochemicals. Solvents and reagent chemicals were from Fisher Scientific, and scintillant fluid was Ultima-Flo M from Packard Instrument Co. (Meriden, CT).

Animals: Female ACI and Sprague-Dawley rats were supplied by Harlan Laboratories (Indianapolis, IN). Animals were housed in an AAALAC-approved animal care facility with a 12 hour light cycle, and provided with food and water *ad libitum*. Estradiol-treated animals received a 20 mg/kg E2 pellet, implanted subcutaneously between the scapulae seven days prior to sacrifice. Animals were anesthetized with ether prior to sacrifice by decapitation.

Mammary Epithelial Cell Isolation and Culture: RMEC were isolated and cultured based on published procedures (38,39). Briefly, mammary glands and associated fat pads were isolated and finely minced, and placed in HEPES-buffered M199 fortified with glutathione, superoxide dismutase, catalase, butylated hydroxyanisole, penicillin/streptomycin, and amphotericin B. Collagenase was added to a final concentration of 0.2% (w/v), and the mixture was incubated at 37° C with shaking. When digestion was judged complete (4-6 hours), cells and organoids were washed and resuspended in M199, treated briefly with 40 μg/ml DNase, and spun through a Percoll gradient to separate epithelial cells. This fraction was washed and resuspended in DMEM/F12. Cells to be stored received 10% porcine serum and 10% DMSO prior to slow freezing and storage in liquid nitrogen. Immediate plating of cells involved the addition of 5 x 10^6 cells to each 10 cm plate, previously coated with collagen, containing 12 ml DMEM/F12 with 10% porcine serum. Cells were cultured at 37° C in humidified air plus 5% CO_2.

Liver Microsomal Preparation: Rat liver microsomes (RLM) were prepared by standard homogenization and centrifugation techniques (40). Microsomes were resuspended in 10 mM PO_4 containing 2 mM $MgCl_2$ and 2 mM dithiothreitol, and stored at -80° C.

Liver Microsomal Metabolism and Analysis: Incubations with RLM were carried out in 10mM PO_4, containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ascorbate, and 5 mM glucose-6-phosphate. [^{3}H]- and [^{14}C]-E2 (0.5 mCi/mmol to 72 Ci/mmol, and 0.5 mCi/mmol, respectively) were added as solutions in DMSO. Final DMSO concentration was 1-3% (v/v). After equilibrating for 3 min at 37° C with shaking reactions were initiated by the addition of an NADPH generating system (NADP and glucose-6-phosphate dehydrogenase). Reactions were terminated by extraction with 2 x 3 vol ethyl acetate. Greater than 99% of labeled materials were isolated by this procedure (data not shown). Extracts were evaporated under reduced pressure, and the residue dissolved in 150 μl of Solvent A (21% methanol, 22% acetonitrile, 57% 0.1% acetic acid in water) for HPLC analysis. HPLC procedures were based on the method provided to us by Dr. Robert Breuggemeier

(Ohio State University). Analysis utilized a Supelcosil C-18 column (5 μ, 4.6 mm x 25 cm) with a linear gradient of Solvent A and Solvent B (40% acetonitrile, 60% 0.1% acetic acid in water). Detection was by absorbance at 280 nm, and quantitation was based on radiometric detection using a Radiomatic A505 detector (Packard Instrument Co., Meriden, CT). Initial product identification was based on co-chromatography with authentic standards of E1 and hydroxylated E1 and E2 metabolites.

RMEC Metabolism and Analysis: Near-confluent primary cultures of RMEC were given 12 ml of DMEM/F12 with 10% porcine serum containing 1 μM [^{3}H]-E2 (160 mCi/mmol). E2 was added as a solution in DMSO. Final DMSO concentration was 0.1% (v/v). After 24 hrs at 37° C in humidified air plus 5% CO_2 the medium was removed, cells were washed with 3 ml medium which was added to the removed medium, and the combined medium was extracted with 3 x 2 vol ethyl acetate. The pooled extracts were evaporated under a reduced pressure and redissolved in 500 μl solvent A. HPLC analysis was as described for microsomal studies, but with an extended elution with Solvent B to allow for the elution of O-methylated metabolites.

Results and Discussion

The original proposal and Statement of Work did not include the comparative studies of the ACI and the Sprague-Dawley rat strains, nor did it include the examination of liver microsomal metabolism. The rationale for the inclusion of the Sprague-Dawley rat for comparison with the ACI rat was discussed in the final paragraph of the Introduction to this Progress Report. The driving force behind the decision to compare two strains is clearly to allow for the assessment of differences in E2 metabolism and actions in two strains which differ markedly in their sensitivity to E2 carcinogenicity. This comparison strengthens the association between observed differences in E2 disposition and actions and the ultimate endpoint, the development of mammary cancer. The rationale for the inclusion of liver microsomal studies should be equally clear. Studies of E2 metabolism by RLM initially were carried out in order to assess the performance of the extraction and analytical procedures to be employed for the proposed studies in the RMEC system. This included the determination of our ability to detect and quantify E2 metabolism at concentrations of the hormone approaching normal plasma levels. It was in the course of these preliminary studies that the profound differences in E2 metabolism between the two strains were first observed. As we present and discuss below, the potential importance of this strain difference necessitated a complete characterization of this difference. This characterization is nearly complete, and the findings will be submitted for publication later this year.

Liver Microsomal Metabolism of Estradiol: Microsomal preparations have been isolated from female ACI and Sprague-Dawley rats and their ability to metabolize labeled E2 has been determined. Both pre- and post-pubertal rats have been studied (*i.e.* 6 weeks and 13 weeks of age, respectively) to determine if puberty has an effect on E2 metabolism. In addition, the effect of treatment of the animals with exogenous E2 on microsomal activities was tested. The initial study for each microsomal preparation was to determine the range of linearity of E2 metabolism as a function of incubation time and of protein concentration. Beginning with E2 at 30 μM, all preparations showed linear increases in metabolism from 10 to 60 min of incubation time with protein concentrations between 0.3 and 1 mg/ml (data not shown). At higher protein concentrations (*i.e.* 2 to 3 mg/ml) increasing metabolism deviated from linearity after 20 min, due to substrate depletion. Parallel incubations containing [^{3}H]-labeled and [^{14}C]-labeled E2 yielded essentially identical results, indicating that loss of tritium from E2 was not a problem. Based on these initial studies, 1 mg/ml microsomal protein and incubation times of either 10 or 20 min were adopted as the standard conditions for following studies.

Investigation of the kinetics of E2 metabolism yielded surprising and provocative results. At E2 concentrations above 3 μM the dominant pathway in both strains was that catalyzed by 17β-hydroxysteroid dehydrogenase, converting E2 to E1 (Fig.1, Panels A and C). 2-Hydroxy-E2 and E1 catechol were the only other metabolites detected. (Note- 2-hydroxy-E1 and 4-hydroxy-E1 are not resolved by the HPLC protocol utilized. The term "E1 catechol" denotes an indeterminate product consisting of 2- and/or 4-hydroxy-E1.) At lower E2 concentrations, however, a qualitative change emerged in E2 metabolism by ACI preparations. Although 2-hydroxy-E2, E1, and E1 catechol were still observed, 4-hydroxy-E2 became the major product (Fig. 1, Panel D). At low E2 concentrations Sprague-Dawley RLM did not make detectable amounts of 4-hydroxy-E2, but instead produced 2-hydroxy-E2, E1, and E1 catechol(Fig. 1, Panel B).

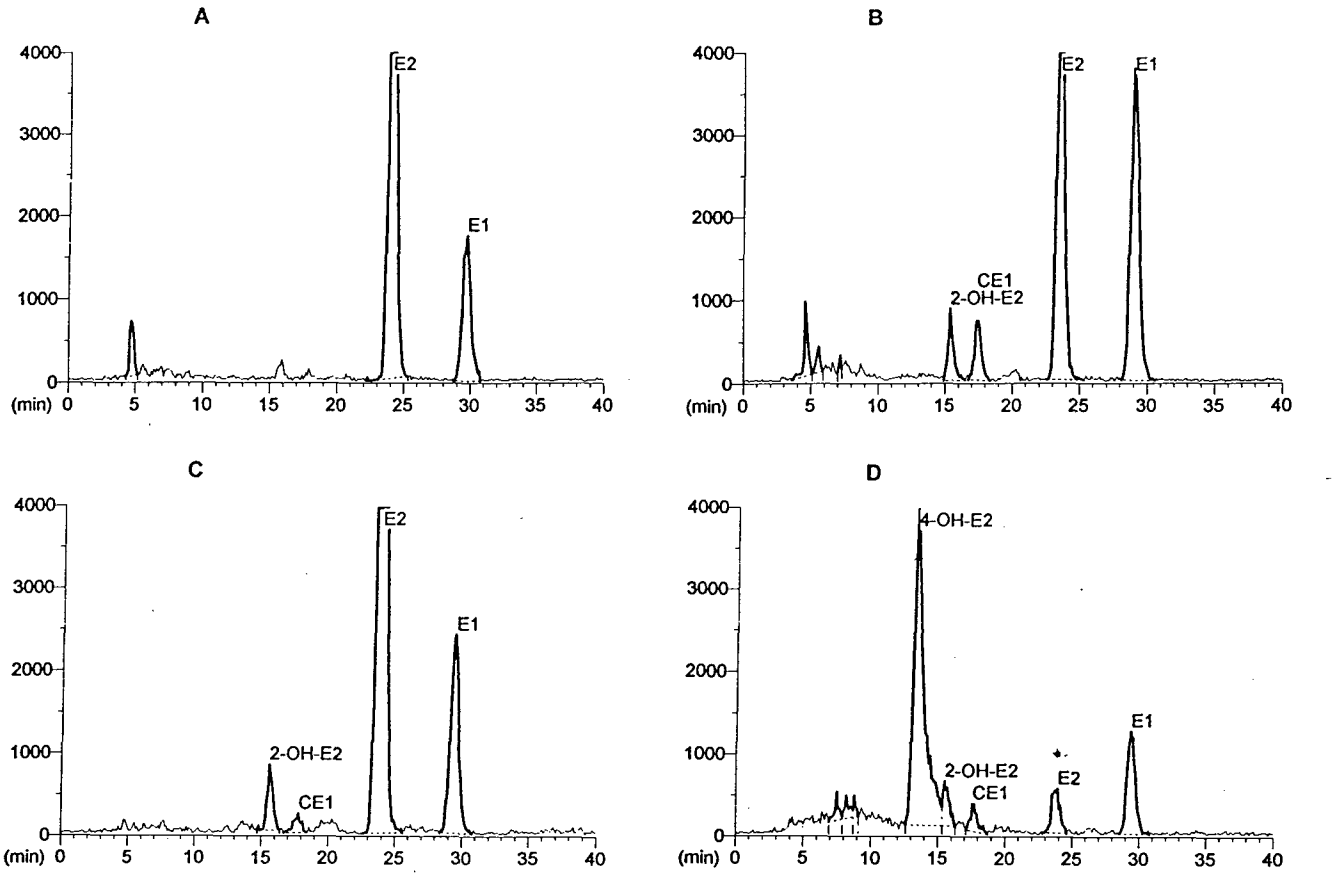

Figure 1. Radiometric HPLC Chromatograms of E2 Metabolism by RLM. Representative chromatograms are shown from incubations of [^{3}H]-E2 with RLM (1 mg microsomal protein ml^{-1}) for 20 min. These data were generated using RLM from post-pubertal rats. Similar results were obtained with pre-pubertal animals. Extraction and analysis was as described in **Experimental Methods**. **Panel A**, Sprague-Dawley RLM, 30 μM E2. **Panel B**, Sprague Dawley RLM, 9 nM E2. **Panel C**, ACI RLM, 30 μM E2. **Panel D**, ACI RLM, 9 nM E2. E2, estradiol; E1, estrone; 4-OH-E2, 4-hydroxyestradiol; 2-OH-E2, 2-hydroxyestradiol; CE1, catechol estrone.

The nature of the enzymes catalyzing the oxidation of E2 was investigated by altering the cofactors present in the incubations and by employing clotrimazole, a broad inhibitor of cytochrome P450-dependent reactions. Reactions were carried out using 1 μM E2 and were extracted 20 min after the addition of cofactors. The results obtained with post-pubertal ACI-derived RLM are shown in Table 1. Similar results were obtained with Sprague-Dawley RLM (data not shown).

Table 1. Modulation of E2 Oxidation: Cofactors and Cytochrome P450 Inhibition

Cofactor	Clotrimazole	4-Hydroxy-E2	2-Hydroxy-E2	E1
NADPH generating system	0 μM	19.0 ± 0.8 pmol min^{-1} mg^{-1}	6.8 ± 0.3 pmol min^{-1} mg^{-1}	8.7 ± 2.2 pmol min^{-1} mg^{-1}
NADPH generating system	10	3.5 ± 0.4	2.8 ± 0.4	20.5 ± 0.5
NADP	0	6.4 ± 0.2	1.4 ± 0.02	30.2 ± 1.9
NAD	0	0	0	50 ± 0

Clearly the aromatic hydroxylation of E2 is NADPH-dependent, as would be expected for a cytochrome P450-

dependent reaction. These reactions are not supported by NAD, and the modest support in the presence of 1 mM NADP may represent *in situ* reduction of this potential cofactor, perhaps by 17β-hydroxysteroid dehydrogenase . The potent inhibition of E2 hydroxylation by clotrimazole also supports the role of cytochrome P450 in the reactions. In contrast, the oxidation of E2 to E1 is supported almost equally by NAD and NADP. Not only is E1 formation not inhibited in the presence of clotrimazole, this reaction is markedly stimulated when the competing aromatic hydroxylation pathways are inhibited. This is consistent with conversion of E2 to E1 catalyzed primarily by 17β-hydroxysteroid dehydrogenase rather than by a P450-dependent reaction.

Analysis of product formation as a function of E2 concentration generated the following kinetic constants:

Table 2. Kinetic Constants for E2 Metabolism by RLM from Post-pubertal Animals.

Metabolite	Sprague-Dawley		ACI	
	K_m	V_{max}	K_m	V_{max}
4-Hydroxy-E2	Not Detected	Not Detected	0.8 ± 0.3 μM	18.8 ± 1.6 pmol min^{-1} mg^{-1}
2-Hydroxy-E2	3.3 ± 1.3 μM	5.8 ± 1.8 pmol min^{-1} mg^{-1}	4.2 ± 0.8	33.9 ± 2.1
E1	14.8 ± 0.8	386 ± 19	38.2 ± 1.8	414 ± 12

The kinetic constants derived from these data clearly establish 17β-hydroxysteroid dehydrogenase as a low affinity, high capacity enzyme for E2 metabolism, with the cytochrome P450-dependent hydroxylation pathways exhibiting higher affinities and lower capacities. Two other key points are suggested by these data. The first is that the ACI rat liver has higher activities of the P450s involved in aromatic hydroxylation of E2 than does the Sprague-Dawley liver. The second, and potentially more important point is derived from a comparison of the K_m values. The 4-hydroxylation of E2 appears to be catalyzed by a different enzyme than is the 2-hydroxylation. This suggests that the ACI rat expresses a hepatic P450 different from those expressed in the Sprague-Dawley liver. This possibility will be addressed by a combination of antibody inhibition studies with anti-P450s in incubations of RLM with E2, and by subsequent Western analysis of RLM from the two strains. If a P450 phenotype or polymorphism is responsible for this difference in hepatic E2 metabolism, then similar analysis will be performed on RMEC-derived samples to assess the expression of the P450s involved.

The effects of E2 administration on the activities of RLM also were determined in the two strains. These data are not yet complete, but preliminary observations suggest that E2 treatment does affect RLM activities. E2 treatment approximately doubles the formation of 4-hydroxy-E2 in the ACI RLM relative to control, and results in detectable amounts of this metabolite in incubations with Sprague-Dawley RLM. The amount of 4-hydroxy-E2 formed by E2-induced Sprague-Dawley liver still is less than 50% of the amount produced by control ACI RLM. 2-Hydroxylation of E2 is increased by about 2-fold in the ACI liver, and about 3-fold in the Sprague-Dawley. E1 formation is slightly suppressed by E2 treatment in both strains. In summary, E2 treatment appears to induce the cytochrome P450s responsible for both the 2- and 4-hydroxylation of E2, but this effect is more pronounced for the isoform responsible for the 2-hydroxylation. The slight suppression of E1 formation probably results from increased competition for E2 due to the increased levels of P450s. When these studies are complete, kinetic constants will be in hand for the formation of the three major products from E2 in two strains, pre- and post-pubertal, and with and without E2 induction. Western analysis of these eight microsomal preparations will be performed to independently test the validity of these preliminary interpretations.

Isolation and Culture of RMEC: The primary system to be studied, however, is not RLM, but rather RMEC. We have successfully isolated and cultured RMEC from ACI rats, using the procedure of Richards *et al.* (40,41). The epithelial cell fraction, purified using Percoll gradient centrifugation, yields approximately 7 x 10^7

cells/rat. These cells grow nicely on a collagen-coated surface. Morphological examination of these cells, however, indicates that they are about 65% myoepithelial cells, as opposed to the lumenal epithelium. Lumenal epithelial cells form proliferating colonies of cuboidal cells, surrounded by the elongate myoepithelial cells. The clear segregation and easy differentiation between these different cell types can be used to our advantage. The mixed cell population allows for the contribution of different cell types from the mammary gland to the overall metabolism of E2 and of DMBA, which more closely models the *in vivo* state of the intact tissue than would a pure lumenal epithelial preparation. During the analysis of effects on proliferation and microtubule integrity, however, the immunohistochemical endpoints can easily be assigned to individual cell types. This is an important advantage in the model, since most mammary tumors are derived from lumenal epithelial cells, rather than myoepithelial cells (4).

Estradiol Metabolism by RMEC: We have performed one study of E2 metabolism by RMEC. Primary cultures were exposed to 1μM [^{3}H]-E2 for 24 hrs, at which time the medium was removed and organo-soluble materials were analyzed by radiometric HPLC. Significant oxidation of E2 was observed, with E1 as the major product. Given the relatively high concentration of E2 employed, and the lack of reducing agents such as ascorbate in the medium, the absence of observed catechol metabolites is not surprising. The key point established by this study is that cultured RMEC are indeed able to oxidize E2.

Proposed Changes in Statement of Work: Obviously the study of E2 metabolism has become far more involved than was originally intended. As is the case in good science, however, the reason for this expansion has been the knowledge gained from experiments performed. The association of 4-hydroxy-E2 formation with sensitivity to E2 carcinogenicity, and the possible involvement of a P450 isoform unique to the ACI rat, is too promising a lead to ignore. This alteration in the course of the studies will necessitate some changes in the Statement of Work. Specifically, it seems prudent to focus on E2 as a carcinogenic estrogen, and to carry out studies of DMBA as a non-estrogenic mammary carcinogen, but to minimize the investigations of DES and ethinyl estradiol as additional carcinogenic estrogens. This change should allow the remaining studies to be performed on schedule and within the available budget.

Conclusions

Profound differences in the hepatic microsomal metabolism of E2 exist between female ACI and Sprague-Dawley rats. Although metabolite profiles from the two strains are virtually identical at micromolar E2 concentrations, the divergence becomes apparent as the substrate concentration drops into the nanomolar range, and is most pronounced at 3 and 9 nM, the lowest concentrations tested. These concentrations approach the physiological plasma concentrations of E2. At these lowest concentrations, 4-hydroxy-E2 is the major product from the ACI rats, whereas E1 is the major product from the Sprague-Dawley. Although both products are estrogenic, 4-hydroxy-E2 is very active in the alkylation of proteins and nucleic acids, and has been proposed to be the ultimate carcinogenic metabolite of E2. Given the exclusive formation of this key metabolite in the rat strain which is most sensitive to the carcinogenic effects of E2 strongly supports a role for this difference in E2 disposition in the differential responses of the strains. Further studies in the liver system will allow for the identification of the cytochrome P450 isoforms responsible for this difference. All results will then be applied to the study and interpretation of E2 metabolism in mammary cells from these rat strains. These studies within the target tissue and cell type will provide a clear picture of *in situ* metabolism of E2 and its potential role in carcinogenicity.

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